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During the second year of the funding period we have investigated the affect of AP-1 blockade on the growth of normal, immortal, and fully malignant breast cells. These studies have demonstrated that the growth of normal and immortal cells is suppressed by AP-1 blockade, while the growth of breast cancer cells is not suppressed. We have also generated breast cancer cell lines which express the AP-1 inhibitior, TAM67, under inducible conditions. The growth of these cancer cells was shown to be insensitive to AP-1 blockade, consistent with our previous results. These cell lines are necessary reagents to address studies proposed in specific aim 2 and specific aim 3 for months 25-48. In the following years of funding we will utilize these reagents to investigate the role of AP-1 transcriptional activity in growth factor-dependent proliferation and oncogene-induced transformation of breast cells.

These studies have demonstrated an involvement of AP-1 transcription complexes in regulating human breast cell proliferation at different stages of the transformation process. The results from these studies and will provide the foundation for future efforts to develop agents which interfere with AP-1 signaling pathways. Such agents may be useful chemopreventive agents to block breast carcinogenesis.

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FOREWORD

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Powel H Brown 9/28/98

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INTRODUCTION

Breast cancer is the most common malignancy in women, and the leading cause of death for women between the ages of 40 and 55 in this country (1). Even with aggressive mammographic screening, adjuvant chemotherapy, and intensive therapy for existing cancer, many of the women who develop breast cancer will die from it. Thus, more effective prevention strategies and treatments are urgently needed.

Unfortunately, little is known about the specific molecular events, which cause the progressive transformation of human breast epithelial cells to malignant breast cancer. Studies of model systems of cancer have revealed that multiple steps are involved in carcinogenesis, including tumor "initiation" and "promotion" events (2). Mutations and deletions within tumor suppressor genes may represent the molecular equivalent of breast cancer "initiation" events (3,4). However, the molecular mechanism of breast tumor "promotion" is poorly defined. In model systems (5), classic tumor promoters induce the proliferation of initiated cells, leading to the progressive outgrowth of fully malignant cells. Such tumor promoters typically activate signal transduction pathways to stimulate cellular proliferation. In human breast cells, the overproduction of growth factors, or aberrant stimulation of growth factor receptors, may be responsible for the promotional phase of breast carcinogenesis. Growth factors important for mammary epithelial cells, such as estrogen, EGF, TGF-a, and the IGFs, may all represent tumor promoters of human breast cancer. Thus, drugs which inhibit the ability of estrogen to activate the estrogen receptor (tamoxifen and other antiestrogens) are used to treat breast cancer, and other drugs which block growth factor receptors, such as antibodies specific for the epidermal growth factor receptor and the Her2/neu receptor, have been shown to inhibit breast cancer cell proliferation (9-11) and are now being tested in clinical trials for the treatment of breast cancer. However, inhibition of individual signal transduction pathways may ultimately be ineffective, since multiple different signal transduction pathways can stimulate breast cell proliferation. It may be more effective to inhibit signal transduction at a more distal point in the cascade, where multiple mitogenic signals converge. Since transcription factors, the nuclear proteins which control DNA transcription and gene expression, are the most distal components of these converging mitogenic signal transduction pathways, they may be ideal targets for new therapeutic agents.

A key family of transcription factors transducing multiple mitogenic signals is the AP-1 family. These transcription factors are complexes of DNA-binding proteins made up of dimers of Jun and Fos proteins, which bind DNA at specific AP-1 sites and regulate the transcription of AP-1-dependent genes. AP-1 transcription factors are expressed in most cell types, and are activated by specific kinases, such as the mitogen-activated and stress-activated kinases, which are themselves activated by diverse signals such as growth factor stimulation, exposure to UV light, oxidative stress, tumor promoters such as TPA, or oncogene overexpression or activation (reviewed in 6). Thus, AP-1 is a central component of many signal transduction pathways in a variety of cell types.

Previous studies showed that the AP-1 transcription factor family is critical for growth factor induced proliferation of fibroblasts (12,13). In addition, we (14,15) and others (16) have shown that AP-1 is also critical for oncogene-induced transformation of fibroblasts. Specifically, we have demonstrated that AP-1 is critical for the cotransformation of primary rat embryo cells by *ras+jun*, *ras+fos*, or *ras+SV40 T antigen* (14), while others have shown that AP-1 is critical for the transformation of NIH3T3 cells by single oncogenes such as *ras*, *raf*, *abl*, and *mos* (16). Thus, AP-1 is a central regulator of transformation as well as mitogenic signaling.

While the role of AP-1 has been extensively studied in fibroblasts, relatively few studies of the function of AP-1 have been performed in epithelial cells and thus the exact role of this transcription factor family in controlling the proliferation and transformation of epithelial cells is not known. Previous studies from our lab and others have demonstrated that the Jun and Fos family members are expressed in human breast cancer cells, and are activated by a variety of important growth factors for

these cells, such as EGF, TGFa, and the IGFs. Studies from other laboratories have also suggested that hormones such as estrogens and retinoids can modulate AP-1 transcriptional activity in breast cells. More recent studies suggest that ER and AP-1 interact to regulate the expression of certain estrogen and/or tamoxifen regulated genes (17). AP-1 complexes may be involved in regulating transcription of the ER gene as well (18). These results suggest that the AP-1 complex may be involved in controlling proliferation of human breast cells; however, definitive studies demonstrating that AP-1 is critical for either breast epithelial cell proliferation or transformation have not been performed.

To address these questions we are using the extensively studied 184 series of normal human mammary epithelial cells (HMECs) isolated and characterized by Dr. Martha Stampfer (19). These cells were originally isolated from reduction mammoplasties of patients and have a normal karyotype, EGF receptors, and specific cytokeratins, suggesting that they are derived from the basal epithelial cells of the normal breast. These HMECs are primary cells, which will senesce after 15-20 passages. However, by exposing these primary HMECs to the carcinogen benzo(a)pyrene, Stampfer *et al.* (19) have established multiple immortalized lines of HMECs (the 184A1 and 184B5 lines). We are studying these carcinogen-immortalized cells as well as the spontaneously immortalized HMEC line, MCF10A, derived from breast tissue obtained from a patient with multiple fibrocystic nodules (20). This cell line expresses cytokeratins and epithelial mucins consistent with a breast epithelial origin, and has cytologic characteristics of breast luminal ductal cells (21). None of the immortal cells are fully transformed since they are not able to grow in an anchorage-independent fashion, or form tumors in nude mice. Recent reports have demonstrated that these immortalized human mammary epithelial cells can be transformed by specific oncogenes such as activated *ras* (22, 23) or *erbB2* genes (24), or by overexpression of *c-myc* or *SV40 T* genes (16). In particular, MCF10A cells can be transformed by an activated *ras* gene (23), while 184B5 cells can be fully transformed by activated *ras* genes (22), or by overexpression of c-*erbB2* (24). Many of these oncogenes are known to activate AP-1 in fibroblasts, though whether these oncogenes also activate AP-1 in breast epithelial cells is not yet known. If AP-1 is involved in regulating these processes, it might therefore serve as a target for the prevention or treatment of breast cancer. To determine the role of AP-1 in controlling breast cell growth and transformation, we proposed to test the following hypotheses:

1. Human breast epithelial cells at different stages in the carcinogenesis pathway express different levels of the AP-1 transcription factor.
2. Breast epithelial cells at these different stages have different requirements for AP-1 for their growth.
3. AP-1 transcription factor activity is necessary for *in vitro* transformation of human breast epithelial cells.

In our previous report we demonstrated that AP-1 transcription factor expression and transcriptional activity is high in normal mammary epithelial cells and is progressively reduced as breast cells proceed towards malignancy completing **Specific Aim 1**. In this report we describe the results of cell proliferation studies described in **Specific Aim 2** which show that normal breast cells are more dependent on AP-1 for their growth than are breast cancer cells. In addition, we have created the reagents necessary to complete **Specific Aim 3.**

BODY

EXPERIMENTAL METHODS AND PROCEDURES

Primary Cell Cultures and Cell Lines:

Human mammary epithelial cells and cell lines used in these studies (Table 1) include normal HMECs isolated from epithelial organoids of human breast from Clonetics (passages 9-10); normal 184 cells (15); 184A1 and 184B5, nontumorigenic immortal cell lines derived from benzo(a)pyrene-treated 184 cells (19); MCF10A (from Dr. J. Russo), a nontumorigenic spontaneously immortalized HMEC cell line; MCF10AneoT (from Dr. J. Russo, Fox Chase Cancer Center, Philadelphia, PA), a transformed cell line derived from MCF10A transfected by c-Ha-ras ; MCF7 WT (wild-type), a human breast adenocarcinoma cell line; MCF7 Adria, a doxorubicin (Adriamycin)-resistant subclone of MCF7 WT (from Dr. K. Cowan, National Cancer Institute, Bethesda, MD). Cells were grown in the following culture media: MEGM (Clonetics, San Diego, CA) for normal HMECs 184, 184A1, and 184B5 (19,25) DME/F-12 with 5% horse serum and supplements as described (20, 23) for MCF10A and MCF10AneoT [with 400 µg/ml Geneticin (G418), Life Technologies, Inc., Gaithersburg, MD]. and Improved MEM (high zinc option; Life Technologies, Inc.) supplemented with 10% FCS and penicillin/streptomycin for MCF7 WT, MCF7 Adria.

Transfection of Breast Cells:

184, clone 91, 184B5, MDA MB 231, MCF7, and T47-D breast cells were transfected using Fugene 6 reagent (Boehringer Mannheim) while MCF10A and MDA MB 435 breast cells were transfected using the LT-1 transfection reagent (PanVera Corp.) according to manufacturer's recommendations.

Western Analysis:

Equal amounts of total cellular protein extract were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad). Primary antibody used was [rabbit anti-cJun Ab-1 from Oncogene Science (Cambridge, MA)]. Blots were developed using the enhanced chemiluminescence (ECL) procedure (Amersham).

Luciferase Assay to Measure AP-1 Activity:

AP-1 transcriptional activating activity in cells was measured using the enhanced luciferase assay (Tropix) as previously described (27). The cells were transfected with the Col-Z-Luc reporter gene containing the luciferase gene linked to 1100 bp of the human collagenase gene promoter which contains a single AP-1 binding site (TGAG/CTCA) between nt. –73 and –60. Transfected cells were lysed 36 hours after transfection and Luciferase activity was measured with equal amounts of cell extract.

Cell Growth Assays:

Colony Formation Assay

2 X 10^5 cells were co-transfected in 35 mm wells with 0.5 ug of pCMV-ß-gal, and 0.5 ug pZeoSV (Invitrogen), and 5 ug of either pCMV (empty vector) or pCMV-TAM-67. Twelve hours after transfection the cells from each 35 mm well were split into four 35 mm wells. 24 hours after replating one well of cells were harvested and used to measure ß-galactosidase activity to determine transfection efficiency. Zeocin (Invitrogen) was added (final concentration of 400 ug/ml) to the three remaining wells. After two weeks of selection in Zeocin, resistant colonies were stained with crystal violet and counted. Colony counts were normalized for transfection efficiency using the ß-galactosidase activity from each transfection.

Single cell proliferation assay:

Cells were co-transfected as described for the colony forming efficiency assay with 0.5 ug of pCMV-ß-gal and 5 ug of either pCMV (empty vector) or of pCMV-TAM-67. Twelve hours after transfection the cells were trypsinized and replated at cell densities of 0.2 to 1.0 X 10^5 in 100 mm plates. After

approximately three doublings the cells were fixed and stained with X-Gal to detect cells expressing ß-galactosidase *in situ*. Colonies containing blue cells were visualized by light microscopy and scored for the number of blue cells per colony.

Cell proliferation assay of stably transfected Tet-on and Tet-off cell lines
The CellTiter 96^{TM} AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) was used to measure breast cancer cell growth according to the protocol provided by the manufacturer. 1000 to 2000 cells were seeded in a 96 well plate and doxycycline was added (MDA MB435 rtTA-vector or -TAM67 lines) or removed (MCF7 tTA-vector or -TAM67 lines) was added the next day and replaced every other day. A solution containing a 20:1 ratio of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium) and PMS (phenazine methosulfate) was added to the cells for 2 hours at 37^0 C and absorption at 495 nm was determined. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

RESULTS

AP-1 Expression and Activity in Breast Cells:

In **Specific Aim 1** we proposed to determine whether changes in AP-1 expression or activity occur as breast cells progress through different stages of carcinogenesis. Breast cells used in this study are listed in Table 1. We have previously shown that normal human mammary epithelial cells have high basal AP-1 activity, immortal breast cells have an intermediate level of basal AP-1 activity, and breast cancer cells have low basal AP-1 activity. We described these results in the 1997-98 annual report and in a 1997 *Cancer Resarch* publication (Smith *et al.* 28).

Table 1: Breast cells used in this study.

Cells	Name	Source	Phenotype
Normal HMECs:	HMEC-91	Clonetics	Senescent, anchorage-dependent
	184	M. Stampfer	
Immortal HMECs:	184B5	M. Stampfer	Immortal, anchorage dependent
	MCF10A	A. Russo	
Breast Cancer cell lines:	MCF7 WT	K. Cowan	Cancer cells, anchorage-independent
	T47-D	ATCC	and tumorigenic
	MDA MB 231	ATCC	
	MDA MB 435	ATCC	

Effect of Inhibiting AP-1 Transactivating Activity on Breast Cell Proliferation:

In **Specific Aim 2** we proposed to determine whether the growth of breast cells at different stages of tumorigenesis are differentially affected by inhibiting AP-1 activity.

TAM67 inhibits AP-1 activity in normal and malignant breast cells:
To determine whether TAM-67 would inhibit AP-1 activity in the different breast cells, we transfected the luciferase reporter construct, Col-Z-Luc, with increasing amounts of the TAM-67 expression plasmid, pCMV-TAM-67. The results of these experiments are shown in Figure 1. We observed that basal AP-1 activity is significantly inhibited by TAM-67 expression in normal, and immortal breast epithelial cells, and in the breast cancer cell lines MCF7 and MDA MB231 (Figure 1). In some breast cancer cells (T47D and MDA MB435) TAM-67 expression resulted in an increase in AP-1 activity.

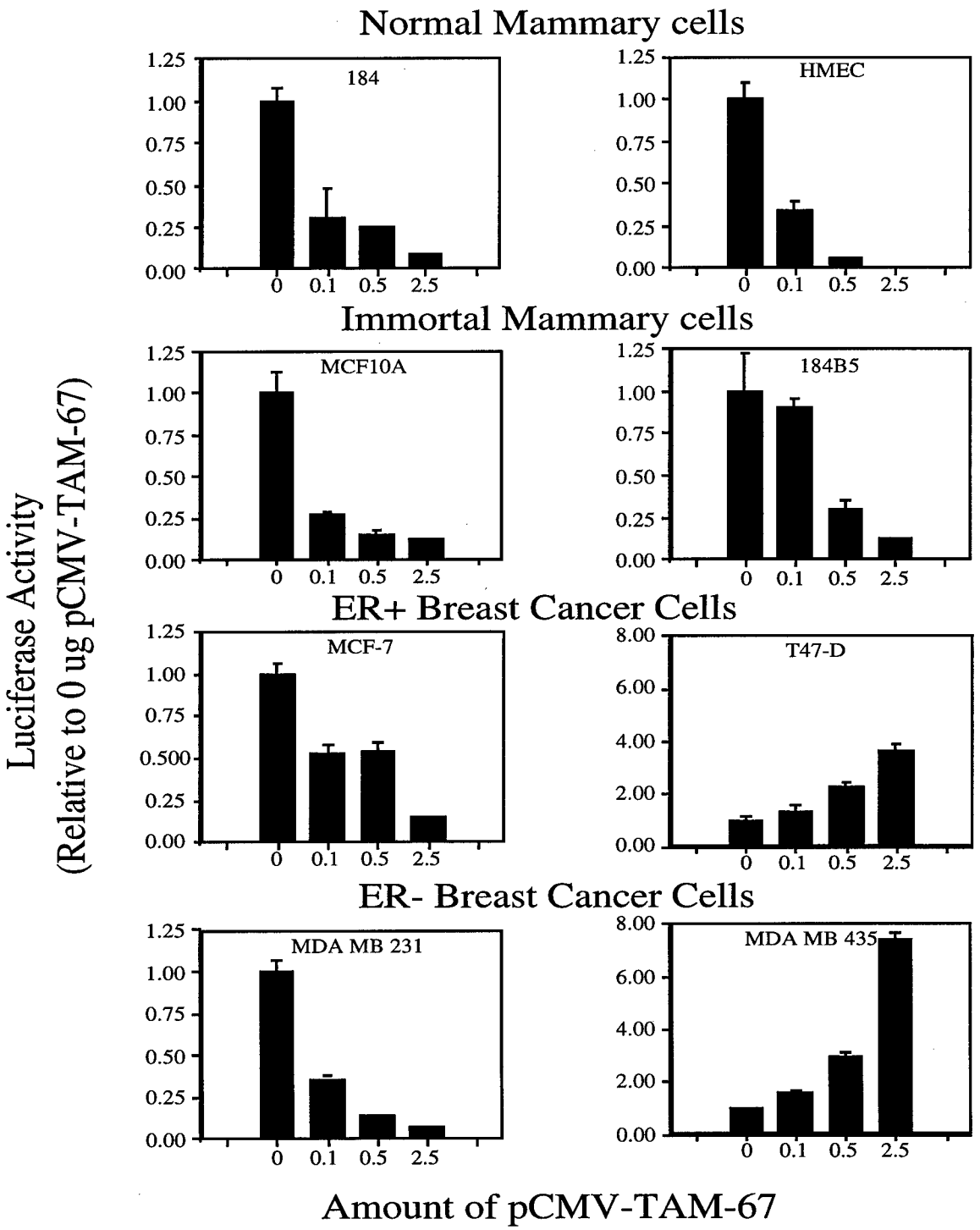

Fig. 1. TAM67 inhibition of Col-Z-Luc activity in breast cells.
Cells were transfected with 1 ug of Col-Z-Luc reporter plasmid plus 0.1, 0.5, or 2.5 ug of the expression plasmid pCMV-TAM67 while maintaining total amount of plasmid at 3.5 ug with the empty expression plasmid pCMV. Transfection procedures were performed as described in "Experimental Procedures and Methods". AP-1 activity was determined by measuring luciferase activity of cell extracts harvested 36 hours post-transfection.

We then investigated the effect of AP-1 blockade on the growth of normal, immortal and malignant breast cells. For these studies we have used three assays: the *colony forming assay* the *single cell proliferation assay*, and growth assays of cells stably transfected with an inducible TAM-67 construct.

Analysis using the colony forming assay:
We investigated whether inhibition of AP-1 transcriptional activity affects HMEC proliferation using a colony forming assay. This assay has been used extensively to demonstrate the effects of tumor suppressors and oncogenes on cell growth. As described in Experimental Methods and Procedures, breast cells were cotransfected with pZeoSV and either pCMV vector or pCMV-TAM-67. The pZeoSV plasmid contains a Zeocin resistance gene allowing selection of transfected cells

Figure 2: Colony formation assay results

A) Colony Formation in Immortal Cells B) Colony Formation in Breast Cancer Cells

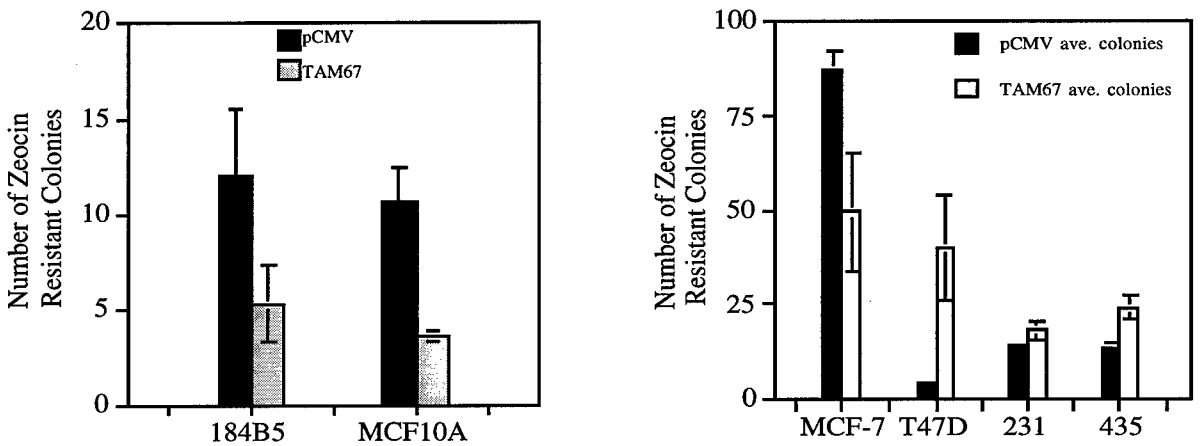

Figure 2: Colony Forming Efficiency of TAM67 Transfected Breast Cells.
Immortalized (A) and malignant (B) breast cells were transfected with 0.5 ug pSVZeo 5ug and either pCMV or pCMV-TAM67 as described in "Experimental Methods and Procedures". After two weeks of selection Zeocin resistant colonies were stained with crystal violet and counted. The data shows the average number of Zeocin resistant colonies from an experiment done in triplicate with error bars representing the SEM. The names of the different cell lines analyzed are given on the x-axis.

Table 2: Summary of colony formation assay results

Cell line	Effect on Colony Formation
Normal HMEC	
HMEC-91	Not determined
184	Not determined
Immortal cells	
184B5	58% reduction
MCF10A	65% reduction
Breast cancer cells	
MCF7	42.5% reduction
T47D	10 fold increase
MDA MB 435	1.7 fold increase
MDA MB 231	1.3 fold increase

We have measured the effect of TAM-67 on colony formation in immortal and malignant breast cells. Normal mammary epithelial cells were not analyzed with this assay because they senesce after a small number of doublings. The results of all these studies are shown in Figure 2 and summarized in Table 2. Colony formation by MDA MB435 and MDA MB231 cells was not inhibited by expression of TAM67 demonstrating AP-1 independent growth which may reflect their lack of dependence on growth factors for proliferation. Inhibition of AP-1 in MCF7 cells decreased colony formation indicating a role for AP-1 transactivating

activity in cell growth while T47D cells showed an increase in colony formation. This observed stimulation of cell growth suggests the possibility that AP-1 complexes have a negative regulatory role in T47D cell proliferation.

Analysis using the single cell proliferation assay (SCPA):
To investigate the effect of AP-1 blockade on normal HMECs and to confirm the above results we used a second growth assay, the single cell proliferation assay previously described by Timchenko *et al.* (32). We used this assay to analyze normal, immortal, and breast cancer cells growth in the presence of TAM-67. The cells were co-transfected with 5 ug of the expression vector pCMV (empty vector) or pCMV-TAM-67 plus 0.5 ug of pCMV-ß-gal. After allowing recovery from the transfection the cells were plated at low cell densities and cultured to allow single cells to grow into small colonies ranging from 1-20 cells. The cells were then fixed and stained *in situ* for ß-galactosidase activity and transfected cells were identified as blue cells by light microscopy. The number of transfected cells observed per colony were scored and presented as a histogram of the percentage of colonies having 1, 2, 3, or more transfected cells per colony.

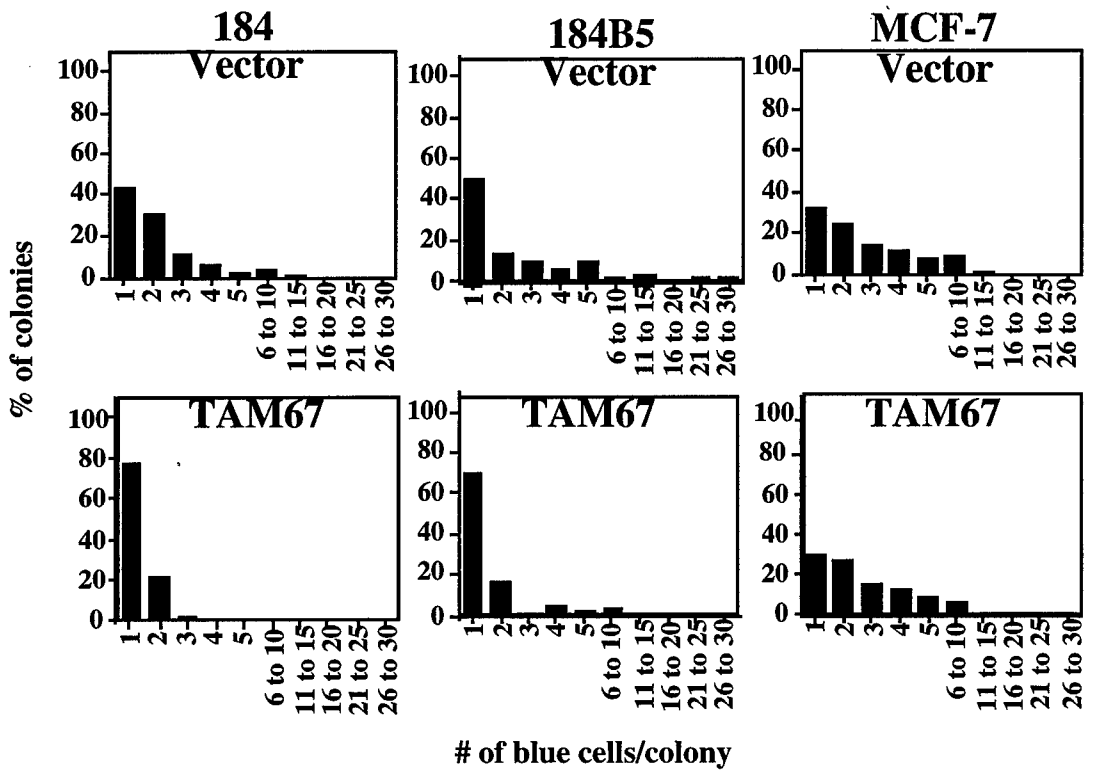

Figure 3: Single Cell Proliferation Assay of Normal, Immortal, and Malignant Breast Cells.
Normal (184), immortal (184B5), and malignant (MCF-7) breast cells were transfected with 0.5ug of pCMV-β-gal and 5ug of either pCMV or pCMV-TAM-67. After approximately three doublings the transfected cells were identified by staining *in situ* for β-galalactosidase activity and the number of transfected cells per colony were counted.

Representative results are shown as histograms in Figure 3 These results demonstrate that the number of b-galactosidase expressing cells in TAM67-transfected normal HMEC and immortal HMEC colonies are reduced as compared to vector-transfected cells, while the number of b-galactosidase expressing cells in TAM67-transfected breast cancer cell colonies was the same as in vector-transfected cells. The complete results are summarized in Table 3 and suggest that the proliferation of normal and immortal breast cells, but not the proliferation of breast cancer cells, is reduced when AP-1 transactivating activity is blocked by TAM-67. The SCPA results are consistent with the colony forming data demonstrating that TAM67 expression inhibited the growth of immortal breast cells while having no

effect on the growth of the two ER-negative breast cancer cells tested. However, the SCPA results demonstrating that TAM67 expression had no effect on MCF7 and T47D proliferation are not consistent with the colony forming data. The observed discrepancy may reflect differences between the ability of TAM67 to inhibit colony formation compared to it's ability to slow the growth of single cells.

Table 3: Summary of single cell proliferation assay data.

Cell line		Growth of transfected cells	P-value*
Normal HMEC			
Clone 91		pCMV-TAM-67<pCMV	0.07
184		pCMV-TAM-67<pCMV	0.0001
Immortal HMEC			
184B5		pCMV-TAM-67<pCMV	0.005
MCF10A		pCMV-TAM-67<pCMV	0.0002
Breast cancer cells			
MDA MB 231	ER- Breast cancer	pCMV-TAM-67=pCMV	0.86
MCF-7	ER+ Breast cancer	pCMV-TAM-67=pCMV	0.65
T47-D	ER+ Breast cancer	pCMV-TAM-67=pCMV	0.90

*Data compared by Wilcoxon rank sum test

Creation of cell lines having inducible TAM-67 expression:
In the grant proposal we described studies to determine whether:

1) AP-1 blockade suppresses mitogenesis induced by specific growth factors, and
2) AP-1 blockade inhibits the transformed-phenotype of breast cancer cells and oncogene-transformed HMECs.

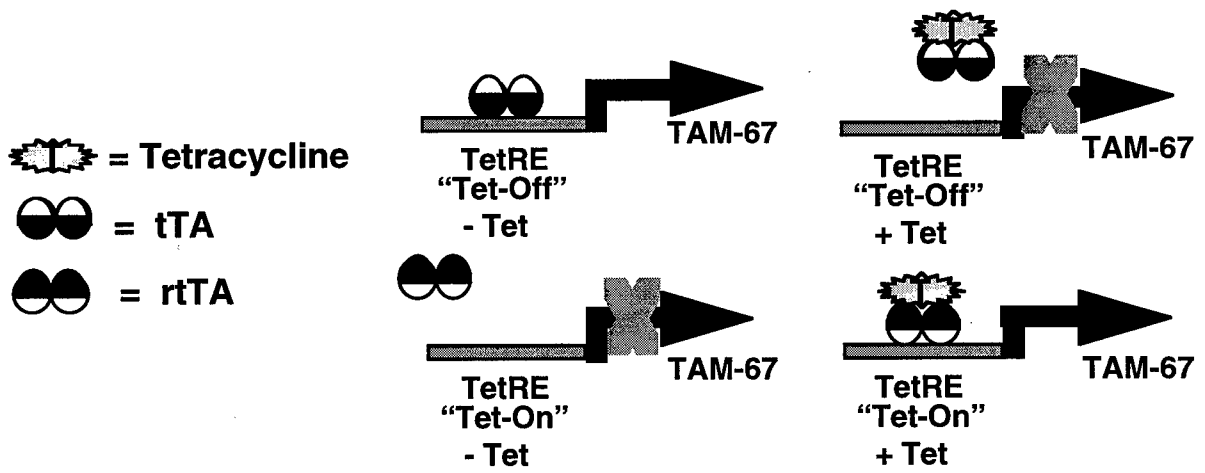

Fig. 4. Tet-off and Tet-on inducible protein expression systems

The Tet-off (top) and Tet-on (bottom) inducible protein expression systems are shown. The Tet-off system utilizes the tetracycline dependent transcriptional repression activity of the tTA protein. The TAM-67 cDNA was cloned downstream of a tTA repressible CMV promoter element (TetRE) in a plasmid having a hygromycin selectable marker. The Tet-on system utilizes the tetracycline dependent transcriptional activation activity of the mutant transcription factor, rtTA. The TAM-67 cDNA was cloned downstream of a rtTA activatable CMV promoter element (TetRE) in a plasmid having a hygromycin selectable marker. With the Tet-off system transfected cells are selected for hygromycin resistance in the presence of doxycyline to maintain repression of TAM-67 expression and protein expression is induced by removal of doxycycline. With the Tet-on system transfected cells are selected for hygromycin resistance in medium lacking doxycycline to maintain uninduced TAM-67 expression and protein expression is induced by addition of doxycycline.

These studies are ongoing, and we have already produced stably transfected cells, which express inducible TAM-67. To produce these clones we have utilized the Tet-on and Tet-off inducible protein expression systems MCF7 and MDA MB435 breast cancer cell lines.

Creation of Tet-on and Tet-off inducible protein expression breast cell lines

The Tet-off system was used for creation of MCF7 TAM-67 cell lines and the Tet-on system was used for the creation of MDA MB435 TAM67 cell lines. MCF7 tTA and MDA MB435 rtTA cells were transfected with expression plasmids containing the flag-tagged TAM67 cDNA inserted downstream of a tetracycline responsive transcriptional promoter and a hygromycin selectable marker as described in Figure 4. Hygromycin resistant colonies were selected under conditions that repress expression of the TAM67 cDNA. Hygromycin resistant colonies were screened for inducible TAM67 protein expression by immunoblotting with anti-cJun antibodies (Figure 5A and 5B). TAM67 basal protein expression was undetectable in both the MCF7 Tet-off and MDA MB435 cell lines. However, after 48

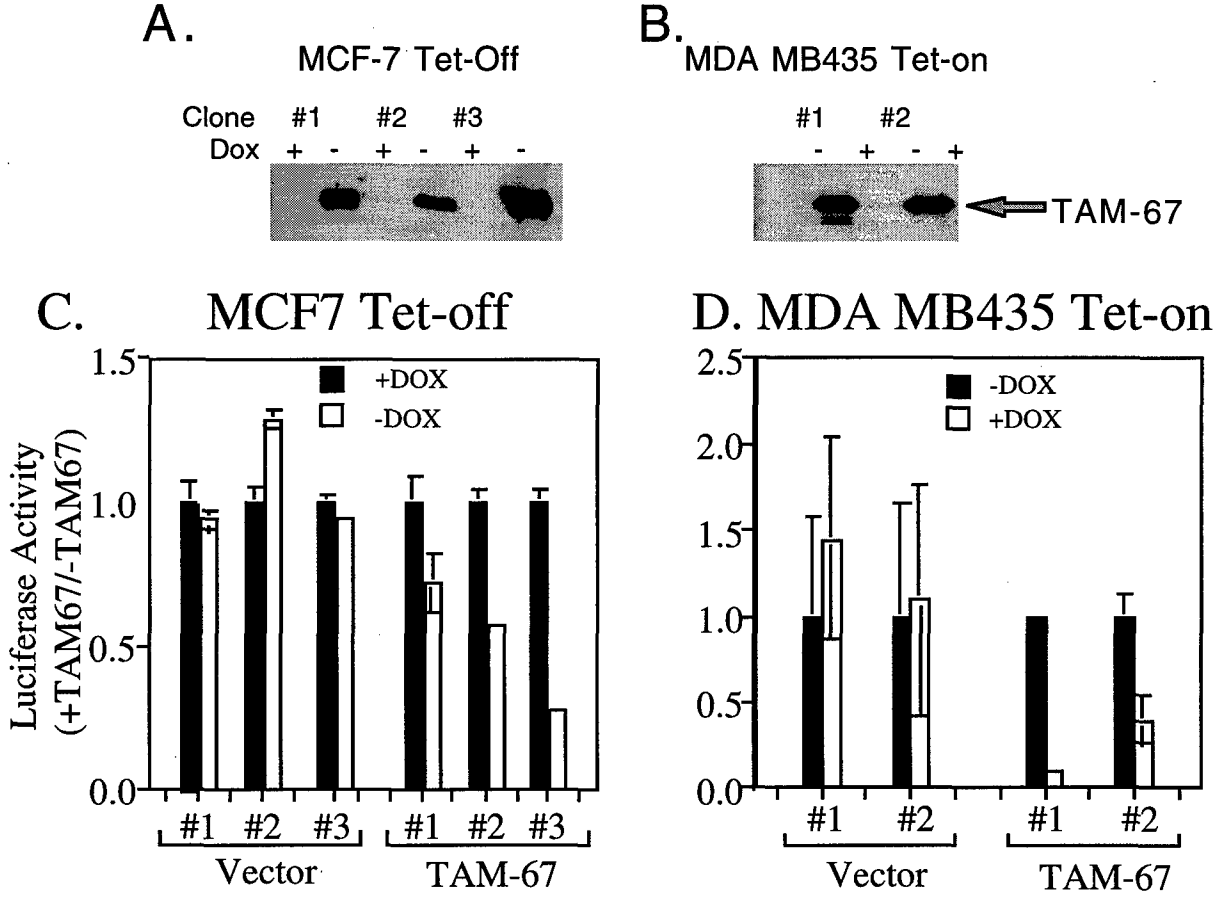

Fig. 5.TAM67 Protein Expression and AP-1 Activity in MCF7 Tet-off and MDA MB435 Tet-on cells.
A and B: Immunodetection of induced TAM67 protein expression in MCF7 Tet-Off (A) and MDA MB435 Tet-On (B) cells. Total cellular protein was extracted 48 hours after induction and equal amounts of protein was analyzed for TAM67 expression using anti-cJun antibody. The TAM67 protein band is indicated with an arrow. C and D: Inhibition of TPA induced AP-1 activity was determined by measuring the effect of TAM67 protein induction on the activity of the transfected AP-1 dependent luciferase reporter plasmid, Col-Z-Luc. Cells were transfected with 1 ug of Col-Z-Luc for 12 hours and split into two plates, with or without doxycycline. After 36 hours of incubation the cells were treated with TPA for 4-6 hours at which time cell extracts were made and luciferase activity was measured. The data are presented as luciferase activity in the presence of TAM67 relative to luciferase activity in the absence of TAM67.

hours of induction a strong signal corresponding to TAM67 was observed in all of the clones which was not present in any vector transfectants (data not shown). These clones were then analyzed for functional activity of the induced TAM67 protein.

The functional activity of the inducible TAM67 proteins was determined by analyzing inhibition of TPA induced AP-1 transactivating activity (Figure 5C and 5D). Cells were transfected with the AP-1 reporter plasmid, Col-Z-Luc, which contains an AP-1 dependent TPA response element upstream of the luciferase gene. The cells were split 1:2 following transfection incubating half of the cells in medium containing doxycycline while incubating the other half of the cells in normal medium. After 36 hours of induction the cells were treated with TPA for 4-6 hours. Under these conditions AP-1 activity is induced more than five fold in both MCF7 Tet-off and MDA MB435 Tet-on. In both MCF7 Tet-off and MDA MB435 Tet-on vector clones the TPA induced activity is the same when incubated in doxycycline containing medium as when incubated in normal medium (Figure 5C and 5D). In contrast, in cells which express TAM67, AP-1 activity is reduced. MCF7 Tet-off cells incubated in the absence of doxycycline induced TAM67 expression (Figure 5C) and TPA induced AP-1 activity was reduced when compared with the same cells grown in the presence of doxycycline which repressed the expression of TAM67. Similarly, when MDA MB435 Tet-on cells were treated with TPA under conditions of TAM67 expression, (i.e. in the presence of doxycycline), induced AP-1 transactivating activity was reduced compared to cells that were not expressing TAM67. Note that these results are in contrast to our results in the transiently transfected cells which showed TAM67 caused an increase in AP-1 activity. It should be noted in our screens for stable clones which express TAM67 some clones which expressed TAM67 showed increased AP-1 activity upon induction by doxycycline.

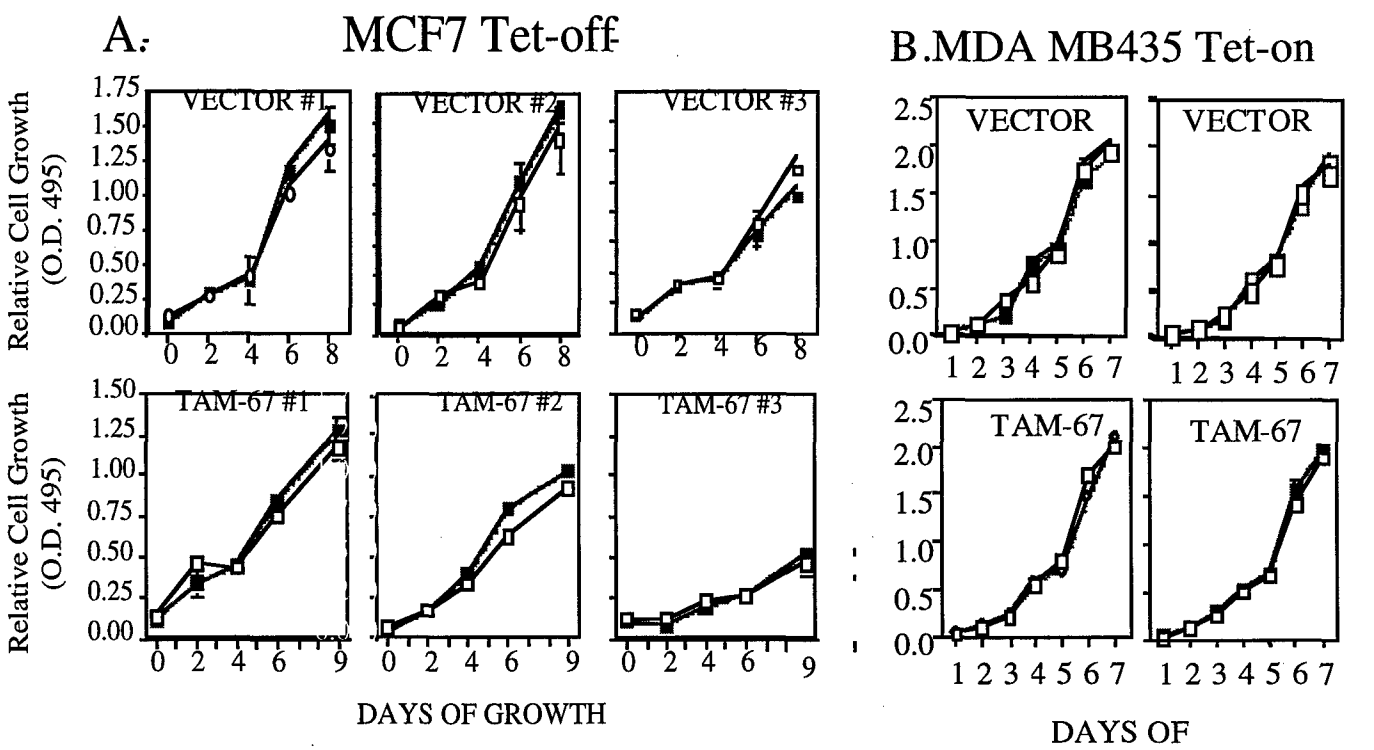

Fig. 6. Effect of induced TAM67 expression on the growth of MCF7 and MDA MB435 breast cancer cells. Growth of MCF7 Tet-off vector and TAM67 clones (A) and MDA MB435 Tet-on vector and TAM67 clones (B) was determined in the presence (filled squares) and absence (open squares) of doxycycline. Every two days cell growth was measured as an increase in the O.D. 495 after incubation with medium containing a MTS/PMS substrate as described in "Experimental Methods and Procedures".

These differences likely arise because of differences in the expression in other Jun and Fos family members or expression of co-activators, as discussed in the discussion below. Taken together the data presented in figure 5 demonstrate that MCF7 Tet-off TAM67 clones and MDA MB435 Tet-on TAM67 clones express inducible TAM67 which causes inhibition of AP-1.

To determine whether AP-1 transactivating activity is required for anchorage dependent growth of breast cancer cells we tested these inducible TAM67 clones in cell proliferation assays (Figure 6). We followed the increase in the cell population grown in the presence and absence of doxycycline using the colorimetric MTS cell proliferation assay. Optical density at 495nm was measured over a period of seven to nine days changing the medium every two days. As shown in Figure 6, the growth of MCF7 and MDAMB 435 cells is not affected conditions, which induce TAM67 protein and inhibit AP-1 activity. These results show that AP-1 blockade does not affect the growth of these breast cancer cells, and are consistent with the results from the colony forming and single cell proliferation assays.

In **Specific Aim 3** we proposed to determine whether inhibition of AP-1 activity can prevent the *in vitro* transformation of immortalized breast cells. For these experiments we will investigate how AP-1 blockade affects the transformed phenotype of immortalized breast cells previously transformed with oncogenes such as c-Ha-ras or c-erbB2. We will also investigate whether AP-1 blockade prevents the *de novo* oncogene-induced transformation of immortalized breast cells.

To investigate whether AP-1 blockade affects the transformed phenotype of oncogene-transformed cells, we will stably transfect ras- or erbB2-transformed 184B5 and MCF10A cells with TAM67 and measure the effect of TAM67 expression on the growth and invasion of these cells. To investigate whether AP-1 blockade prevents transformation, we will isolate clones of 184B5 and MCF10A which express inducible TAM67 to examine whether the presence of TAM67 affects the ability of these cells to become transformed by oncogenes such as ras and erbB2. Through these sets of experiments we will determine how AP-1 blockade affects the transformation process.

DISCUSSION

We have previously demonstrated that normal human breast cells express high basal and induced levels of AP-1 activity and that breast cancer cells express relatively low levels of AP-1 activity. The studies in this report were performed to determine whether these different levels of AP-1 in normal and malignant breast cells reflect a difference in these cells' dependence on AP-1 for their growth. The above results demonstrate that the growth of normal and immortalized breast cells is inhibited by AP-1 blockade, while the growth of breast cancer cells is not. These findings show that normal and immortalized human breast cells are more dependent on AP-1 for the transduction of mitogenic signals than are breast cancer cells.

These results showing that premalignant breast cells depend on AP-1 for transducing mitogenic signals is consistent with previous reports demonstrating that normal human mammary epithelial cells require peptide growth factors to support their growth. Stampfer *et al.* (15) and Zajchowski *et al.* (40) have demonstrated that peptide growth factors are required for the *in vitro* growth of normal human mammary epithelial cells. In addition, Stampfer*et al.* (34) have shown that 184 normal human mammary epithelial cells, and the 184B5 immortalized cell line derived from these normal cells, are both dependent on TGFα for continued growth. In previous studies supported by this grant (28), we have shown that the high basal level of AP-1 in normal mammary epithelial cells is suppressed by suramin, suggesting that this high level of AP-1 is maintained through the production of autocrine growth factors such as TGFα. All of these studies demonstrate that normal breast cells require peptide growth factors to sustain their growth. The present results extend this observation to show that the activity of the AP-1 transcription factor, a downstream transducer of these peptide growth factors, is critical for the growth of these cells.

Our results also demonstrate that certain breast cancer cells (MCF7, MDAMB231 and MDAMB435 cells) are less dependent on AP-1 for their growth than are normal breast cells. While the AP-1 inhibitor, TAM67, blocked AP-1 activity in these cells, the growth of these cells was unaffected by this AP-1 blockade. The growth of another breast cancer cell line, T47D, was also not inhibited by TAM67. However, in this cell line TAM67 increased AP-1 activity, and did not inhibit AP-1 as it did in the other normal and malignant breast cells. In addition, in the transiently transfected MDA MB435 cells, but not in our inducible stably transfected MDAMB435 clones, we also observed that TAM-67 increased AP-1 activity. Such an increase in AP-1 activity by TAM67 is unusual, since TAM-67 has been shown to inhibit AP-1 in many different cell types including breast, skin epithelial cells, fibroblasts, and myeloid cells. (28,41,42,43). However, Zagariya *et al.* showed that TAM67 increased the activity of the TNFα promoter in response to phorbol esthers in U937 myeloid cells . It is likely that the effect of such a dominant-negative cJun mutant will depend on the expression of other Jun or Fos family members, other dimerizing partners, or coactivators or corepressors, in the particular cell of interest. In the case of T47D and in some MDAMB435 cells, these cells may express other proteins, which interact with TAM67 to allow this complex to activate AP-1 dependent genes.

The growth of breast cancer cells is often dependent on growth factors such as IGFs, TGFα, an estrogen. However, the present results suggest that at least for the breast cancer cell lines tested, activation of AP-1 dependent pathways is not essential for the growth of these cancer cells. While IGFs, TGFα, and estrogen have all been shown to activate the AP-1 transcription factor, it has not previously been known whether the activation of AP-1 is critical for the mitogenic effect of these growth factors. The present results suggest that for the breast cancer cells tested here (MDAMB231, MDAMB435, and possibly MCF7), AP-1 is not critical for transducing these mitogenic signals.

It is possible that breast cancer cells which have genetic alterations in tumor suppressor genes, such as P53 or Rb mutations, or overexpression of oncogenes, such as c-erbB2/her2/neu, c-myc, or cyclin D, no longer require mitogenic signals which are normally transduced by activation of AP-1. The observation that normal human breast cells which lack these genetic alterations require AP-1 activity for their growth, while the cancer cells do not is consistent with this hypothesis. All of the breast cancer cells studied here have known genetic alterations. T47D and MDA MB231 have known P53 mutations, while the 184 normal breast cells, the immortalized cells have been shown to express normal p3. These genetic alterations may disrupt the mitogenic signal transduction pathway at a step distal to AP-1, and thus render these transformed cells resistant to AP-1 inhibitors.

By using these normal, immortalized, and fully malignant breast cells as a model for breast tumorigenesis, we have been able to characterize the specific transcription factors required for growth of breast epithelial cells at different points in the carcinogenesis pathway. The characterization of these critical signal transudation pathways may lead to the identification of novel targets for the treatment or chemoprevention of breast cancer. The results reported here showing that normal breast cells are more dependent on AP-1 for their growth than are breast cancer cells, suggest that agents which inhibit AP-1 or inhibit Jun N-terminal kinases, would be more useful for the prevention of cancer than for the treatment of established cancers.

ONGOING STUDIES

We are continuing our studies of AP-1 inhibition in immortalized HMECs and breast cancer cells. During the following years of the grant we will:

1) Determine whether AP-1 blockade suppresses mitogenesis induced by specific growth factors.

To accomplish these studies we will use cell lines that have inducible expression of the TAM67 protein. We are using the tetracycline inducible system (33) in breast cancer cells and immortalized cells.

We have already isolated MCF7 (ER+) and MDA MB435 (ER-) breast cancer clones which express inducible TAM67. We are now generating immortal breast cells with inducible TAM67 expression that will be used to study the role of AP-1 in growth factor induced mitogenesis. To determine whether AP-1 blockade suppresses growth factor induced mitogenesis, the cells will be grown in serum free medium or medium containing EGF, IGF, heregulin, or estrogen with and without induction of TAM-67. The growth of these cells stimulated with the different growth factors will be measured and growth in the absence and presence of the AP-1 inhibitor will be compared.

2) Determine whether AP-1 blockade inhibits the transformed-phenotype of breast cancer cells and oncogene-transformed HMECs.

The MCF7 Tet-off and MDA MB435 Tet-on cell lines with inducible TAM67 are being used to determine whether AP-1 transactivating activity is required for anchorage independent growth, tumor formation in nude mice, and cancer cell invasiveness *in vitro*.

We have established several clones of 184B5 and MCF10A cells that stably express an activated erbB2 oncogene or an oncogenic ras protein. All of these clones exhibit the transformed phenotype of anchorage independent growth. These transformed HMECs and breast cancer cells will be used to determine whether inhibition of AP-1 transcriptional activity effects the transformed phenotype of breast cells. TAM-67 will be expressed in these cells and then assayed for the ability to grow in soft agar.

PROGRESS RELATIVE TO STATEMENT OF WORK

Specific Aim 1: To determine whether changes in AP-1 expression or activity occur as HMECs progress through different stages of carcinogenesis.
We have completed this specific aim and have completed tasks for months 1-12 listed in the statement of work. The results of these studies were described in our first year annual report, presented at the the "Era of Hope" meeting in Washington, D.C., November, 1997, and published in *Cancer Research* (28). These studies lead to the conclusions that Jun and Fos protein expression and AP-1 activity are high in normal human mammary epithelial cells, and are reduced as breast cells progress toward a more malignant phenotype.

Specific Aim 2: To determine whether growth of HMECs at the different stages is differentially affected by inhibiting AP-1 activity.
The tasks for months 1-24 have been completed and summarized in this report. Preliminary results were presented at the "Era of Hope" meeting in Washingtion, D.C., in November 1997 and the manuscript describing the completed studies is now in preparation. We are now proceeding with experimental tasks for months 25-36, and are now investigating the effect of AP-1 blockade on mitogenesis induced by specific growth factors in breast cells.

Specific Aim 3: To determine whether inhibition of AP-1 activity can prevent the *in vitro* transformation of immortalized HMECs.
We have completed the tasks for months 1-24, and have developed an *in vitro* transformation assay and have isolated oncogene-transformed HMECs and determined their transformed phenotype. We are now measuring the effect of TAM67 expression on the transformed phenotype of these oncogene-transformed cells, and are beginning experiments to investigate how TAM67 will affect the *de novo* transformation of immortalized breast cells (tasks for months 25-48).

CONCLUSIONS

During the second year of the funding period we have investigated the affect of AP-1 blockade on the growth of normal, immortal, and fully malignant breast cells. These studies have demonstrated that the growth of normal and immortal cells in suppressed by AP-1 blockade, while the growth of breast cancer cells is not suppressed. We have also generated breast cancer cell lines which express the AP-1 inhibitor, TAM67, under inducible conditions. The growth of these cell lines were shown to be insensitive to AP-1 blockade, consistent with our previous results. These cell lines are necessary reagents to address studies proposed in specific aim 2 and specific aim 3 for months 25-48. In the following years of funding we will utilize these reagents to investigate the role of AP-1 transcriptional activity in growth factor-dependent proliferation and oncogene-induced transformation of breast cells.

These studies have demonstrated an involvement of AP-1 transcription complexes in regulating human breast cell proliferation at different stages of the transformation process. The results from these studies and will provide the foundation for future efforts to develop agents which interfere with AP-1 signaling pathways. Such agents may be useful chemopreventive agents to block breast carcinogenesis.

REFERENCES

1. Harris, J., Morrow, M. and Bodadonna, G. Cancer of the Breast. *In:* J. Devita VT, H. S and R. SA (eds.), Cancer of the Breast, pp. 1264-1332. Philadelphia: J.B. Lippincott Co., 1993.
2. Bishop, J. The molecular genetics of cancer. *Science*, 235: 305-311, 1987.
3. Tripathy, D., Benz, C.C. Activated oncogenes and putative tumor suppressor genes involved in human breast cancer. *In:* L. Benz (eds.), Activated oncogenes and putative tumor suppressor genes involved in human breast cancer, pp. 15-60. Boston: Kluwer Academic Publishers, 1993.
4. Malkin D., Lee, F.P., Strong, L.C., Fraumeni, J.F., Nelson , C.E., Kim, D.H., Kassel J., Gryka, M.A., Bischoff, F.Z., Tainsky, M.A., Freind, S.H. Germ line p53 mutation in a familial syndrome of breast cancer sarcomas and other neoplasms. *Science*, 250: 1233-1238, 1990.
5. Berenblum, I., Shubik, P. The role of croton oil applications, associated with a single painting of a carcinogen, in tumour induction of the mouse's skin. *Br J Cancer,* 1: 379-383, 1947.
6. Baselga, J., and Mendelsohn, J. The epidermal growth factor receptor as a target for therapy in breast carcinoma. *Breast Cancer Res. Treat.* 29:127-138, 1994.
7. Sarup, J.C., Johnson, R.M., King, K.L., Fendly, B.M., Lipari, M.T., Napier, M.A., Ullrich, A., Shepard, H.M. Characterization of an anti-p185(Her2) monoclonal antibody that stimulates receptor function and inhibits tumor cell growth. *Growth Regulation* 1:72-82, 1991.
8. Drebin, J.A., Link, V.C., Greene, M.I. Monoclonal antibodies specific for the *neu* oncogene product directly mediate anti-tumor effects in vivo. *Oncogene* 2:387-394, 1988.
9. Angel, P. and Karin, M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biocheimica et Biophysica Acta,* 1072: 129-157, 1991.
10. Holt, J.T., Venkat-Gopal, T., Moulton, A.D., Nienhuis, A.W. Inducible production of c-fos antisense RNA inhibits 3T3 cell proliferation. *Proc. Natl. Acad. Sci. USA.* 83:4794-98, 1986.
11. Cozenza, S.C., Yumet, G., Soprano, D.R., Soprano, K.J. Induction of c-*fos* and c-*jun* mRNA at the M/G1 border is required for cell cycle progression. *J. Cell. Biochem.* 55:503-512, 1994.
12. Brown, P. H., Alani, R., Preis, L. H., Szabo, E. and Birrer, M. J. Suppression of oncogene-induced transformation by a deletion mutant of c-jun. *Oncogene, 8:* 877-886, 1993.
13. Brown, P.H., Chen, T., Birrer, M.J. 1994. Mechanism of action of a dominant-negative mutant of cJun. *Oncogene* 9:791-800.
14. Rapp, U. R., Troppmairi, J., Beck, T. and Birrer, M. J. Transformation by Raf and other oncogenes renders cells differentially sensitive to growth inhibition by a dominant negative cJun mutant. *Oncogene* 9:3493-3498, 1994.
15. Stampfer, M., and Yaswen, P. Factors influencing growth and differentiation of normal and transformed human mammary epithelial cells in culture. In: *Transformation of human epithelial cells: molecular and oncogenetic mechanisms.* Eds: Milo, G., Casto, B., Shuler, C. CRC Press, Boca Raton. 1992. pp 117-139.
16. Clark, R., Stampfer, M.R., Milley, R., O'Roarke, E., Walen, K.H., Kriegler, M., Kopplin, J., McCormick, F. Transformation of human mammary epithelial cells by oncogenic retroviruses. *Cancer Res.* 48:4689-4694, 1988.
17. Peach, K., Webb, P., Kuiper, G.G.J.M., Nilsson, S., Gustafsson, J-K., Kushner, P.J., Scanlan, T.S. Differential ligand activation of estrogen receptors ERa and ERb at AP-1 sites *Science* 277:1508-1510, 1997.
18. Tang, Z., Treilleux, I., Brown, M. A transcriptional enhancer required for the differential expression of the human estrogen receptor in breast cancers *Mol.. Cell. Biol.* 17:1274-1280, 1997.
19. Stampfer, M.R., and Bartley, J.C. Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo(a)pyrene. *Proc. Natl. Acad. Sci. USA* 82:2394-2398, 1985.
20. Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson, W.D., Brenz, R., McGrath, C.M., Russo, J., Pauley, R.J., Jones, R.F., Brooks, S.C. Isolation and Characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res.* 50: 6075-6086, 1990.

21. Tait, L., Soule, H.D., and Russo, J. Ultrastructural and immunocytocheimcal characterization of an immortalized human breast epithelial cell line, MCF-10. *Cancer Res.* 50: 6087-6094, 1990.
22. Basolo, F., Elliott, J., Tait, L, Chen, X., Maloney, T., Russo, I., Pauley, R., Momiki, S., Caamano, J., Klein-Szanto, A., Koszalka, M., Russo, J. Transformation of human breast epithelial cells by c-Ha-*ras* oncogene. *Mol. Carcinogenesis* 4:25-35, 1991.
23. Ciardiello, F. McGeady, M.L., Kim, N., Basolo, F., Hynes, N., Langton, B.,C., Yokozaki, H., Saiki, T., Elliott, J., Hasui, H., Mendelsohn, J., Soule, H., Russo, J., Salomon, D.S. Transforming growth factor-a expression is enhanced in human mammary epithelial cells transformed by an activated c-Ha-*ras* protooncogene but not by the c-*neu* protooncogene, and overexpression of the transforming growth factor-a complementary DNA leads to transformation. *Cell Growth and Diff.* 1:407-420, 1990.
24. Pierce, J., Arnstein, P., DiMarco, E., Artrip, J., Kraus, M., Lonardo, F., Di Fiore, P. and Aaronson, S. Oncogenic potential of *erbB-2* in human mammary epithelial cells. *Oncogene*, 6: 1189-1194, 1991.
25. Stampfer, M., Hallowes, R.C., and Hackett, A.J. Growth of normal human mammary epithelial cells in culture. *In Vitro*, 16:415- 425, 1980.
26. Walen, K., and Stampfer, M.R. Chromosome analyses of human mammary epithelial cells at stages of chemically-induced transformation progression to immortality. *Cancer Genet. Cytogenet.*, 37:249-261,1989.
27. Chen, T.K., Smith, L.M., Gebhardt, D.K., Birrer, M.J., and Brown, P.H. Activation and inhibition of the AP-1 complex in human breast cancer cells. *Mol. Carcinog.*, 15:215-226,1996.
28. Smith, L.M., Birrer, M.J., Stampfer, M.R., and Brown, P.H. Breast cancer cells have lower activating protein 1 transcription factor activity than normal mammary epithelial cells. *Cancer Research*, 57:3046-3054, 1997.
29. Schontal, A., Herrlich, P., Rahmsdorf, H.J., and Ponta, H. Requirement for *fos* gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. *Cell*, 54:325-334, 1988.
30. Sistonen, L., Holtta, E., Lehvaslaiho, H., Lehtola, L., and Alitalo, K.J. Activation of the neu tyrosine kinase induces the fos/jun transcription factor complex, the glucose transproter and ornithine decarboxylase. *J. Cell. Biol.*, 109:1911-1919, 1989.
31. Binetruy, B., Smeal, T., and Karin, M. Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. *Nature (Lond.)*, 351:122-127, 1991.
32. Timchenko, N.A., Wilde, M., Nakanishi, M., Smith, J.R., Darlington, G.J. CCAAT/enhancer-binding protein a (C/EBPa) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. *Genes and Dev.*, 10:804-815.1996.
33. Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., Bujard, H. Transcriptional activation by tetracyclines in mammalian cells. *Science* 268:1766-1769, 1995.
34. Stampfer, M.R., Pan, C.H., Hosoda, J., Bartolomew, J., Mendelsohn, J., and Yaswen, P. Blockade of EGF receptor signal transduction causes reversible arrest of normal and immortal human mammary epithelial cells with synchronous reentry into the cell cycle. Exp. Cell. Res., 209: 175-188, 1993.
35. Pfarr, C.M., Mechta, F., Spyrou, G., Lallemand, D., Carillo, S., and Yaniv, M. Mouse JunD negatively regulates fibroblast growth and antagonizes transformation by ras. Cell, 76: 747-760, 1994.
36. Nakabeppu, Y., and Nathans, D. A naturally occurring truncated form of fosB that inhibits fos/jun transcriptional activity. Cell, 64: 751-759, 1991.
37. Yoshioka, K., Deng, T., Cavigelli, M., and Karin, M. Antitumor promotion by phenolic antioxidants: inhibition ov AP-1 activity through induction of Fra expression. Proc. Natl. Acad. Sci. USA, 92: 4972-4976, 1995.
38. Kerppola, T.K., and Curran, T. Maf and Nrl can bind to AP-1 sites and form heterodimers with Fos and Jun. Oncogene, 9: 675-684, 1994.
39. Kataoka, K., Noda, M., and Nishizawa, M. Transactivation activity of Maf nuclear oncoprotein is modulated by Jun, Fos, and small Maf proteins. Oncogene, 12: 53-62, 1996.

40. Zajchowski, D., Band, V., Tager, A., Stampfer, M., and Sager, R. Expression of growth factors and oncogenes in normal and tumor-derived human mammary epithelial cells. Cancer Res., 15:7041-7047.
41. Li, JJ., Rhim, RS., Schlegel, R,. Vousden, KH., Colburn, NH. Expression of dominant negative Jun inhibits elevated AP-1 and NF-kappaB transactivation and suppresses anchorage independent growth of HPV immortalized human keratinocytes. Oncogene. 28:2711-2721, 1998
42. Silberman, S., Janulis, M., Schultz, RM. Characterization of downstream Ras signals that induce alternative protease-dependent invasive phenotypes. J Biol. Chem. 28:5972-5935, 1997.
43. Grant, S., Freemerman, AJ., Birrer, MJ., Martin, HA., Turner, AJ., Szabo, E., Chelliah, J., Jarvis, WD. Effect of 1-beta-D-arabinofuranosylcytosine on apoptosis and differentiation in human monocytic leukemia cells (U937) expressing a c-Jun dominant -negative mutant protein (TAM67). Cell Growth Differ 7:603-613, 1996.
44. Zagariya, A., Mungre, S., Lovis, R., Birrer, M., Ness, S., Thimmapaya, B., and Pope, R. Tumor necrosis factor alpha gene regulation: Enhancement of C/EBP-ß-induced activation by c-Jun. Mol Cell Biol. 18:2815-2824, 1998.

ABBREVIATIONS

AP-1	Activating Protein 1
ATCC	American Type Culture Collection
ATF	Activating Transcription Factor
bp	base pairs
CBP	Creb Binding Protein
cDNA	complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
DME	Dulbecco's Modified Eagle
ECL	Enhanced Chemiluminesence
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
FCS	Fetal Calf Serum
Ha-ras	Harvey-ras
HMEC	Human Mammary Epithelial Cells
IGF	Insulin-like Growth Factor
MEGM	Mammary Epithelial Growth Medium
MEM	Modified Eagle Medium
ml	milliliter
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
neo	neomycin transferase
nm	nanometer
O.D.	Optical Density
PMS	phenazine methosulfate
rtTA	reverse tetracycline-controlled transactivator
SEM	Standard Error of the Mean
SV40	Simian Virus 40
tTA	tetracycline-controlled transactivator
Tet	Tetracycline
TetRE	Tetracycline Response Element
TGF	Transforming Growth Factor
TPA	12-O-tetradecanoyl-phorbol-13-acetate
ug	microgram
WT	Wild-type